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SPECIFIC DOUBLE DIFFUSION MICROTECHNIQUE FOR THE DIAGNOSIS OF ASPERGILLOSIS AND PARACOCCIDIOIDOMYCOSIS USING MONOSPECIFIC ANTISERA

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Using experimental reference sera against species-specific antigens of *Aspergillus fumigatus* and *Paracoccidioides brasiliensis* in a microdouble diffusion technique, a simple and specific test for the immunodiagnosis of aspergillosis and paracoccidioidomycosis has been developed. Only sera that produced lines of identity with either one of the bands formed by the anti-C_2 or the anti-E_2 reference sera were considered positive for aspergillosis or paracoccidioidomycosis, respectively. The sensitivity of the diagnostic test was similar to those of the classical double diffusion and the immunoelectrophoresis tests. No false positives were found in sera obtained from patients affected by other mycoses, nor from healthy controls.

The amount of reagents for the specific test was ten fold less than that required by the classical double diffusion test.

Double diffusion in gel (DDG) is the practical of the tests used in the immunodiagnosis of mycotic diseases. Described by Ouchterlony (10), it has been applied by a number of workers to the study of various fungal infections including aspergillosis (12), candidiasis (13, 14) coccidioidomycosis (7), chromomycosis (4), histoplasmosis (6), mycetomas (9), paracoccidioidomycosis (11) and sporotrichosis (2). The method has the special advantage that it is very simple to perform. It is only limited by the risk of false positive reactions, due to the common antigens shared by pathogenic fungi; also, the fact that relatively large quantities of reactants (150 microliter of antigen and serum) must be used, is another limitation. The recent experimental production of monospecific antisera against species-specific antigens of *Aspergillus fumigatus* (3) and *Paracoccidioides brasiliensis* (19) and the existence of micro-variants of the DDG (8) suggested to us the possibility of rendering the test more specific, by combining one of the microtechniques with the use of a reference monospecific antiserum.

The present study was carried out to determine the specificity and the sensitivity of the resulting modified microtechnique in the diagnosis of aspergillosis and paracoccidioidomycosis.

Materials and Methods

Antigens

Crude soluble extracts of *A. fumigatus* (Longbottom strain), and of both morphological phases of *P. brasiliensis* (IHM* 1572 strain), were used. The antigen of *A. fumigatus* was extracted by the method described by Biguet, Fruit, Andrieu and Tran Van Ky (1). In the case of *P. brasiliensis*, the yeast extract was obtained

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according to Restrepo (11) and the mycelial antigen by the technique reported by Yarzábal (18).

Human sera

One hundred and thirty sera were tested by the modified DDG test. They were obtained from: (i) patients with different fungal diseases, all proven by microscopical observation and/or isolation of the fungus in culture, and (ii) normal controls. The first group was composed of sera from patients with aspergilloma (9), systemic candidiasis (6), chromomycosis (3), coccidioidomycosis (4), histoplasmosis (18), lobomycosis (1), mycetomas (4), paracoccidioidomycosis (45), and sporotrichosis (15). All sera were taken before therapy and stored for periods of 1 month to 7 years at –20°C, without preservative.

Experimental Monospecific Antisera

Antisera were prepared against the C_2 antigen of *A. fumigatus* (15), and the E_2 of *P. brasiliensis* (20), using the procedure described by Goudie, Horne & Wilkinson, (5) as modified by Bout *et al.*(3). Briefly, the precipitation arcs corresponding to the chosen antigens were identified and excised from immunoelectrophoresis slides which have been prepared with the crude antigen and the corresponding homologous antisera. The latter had been previously rendered species-specific by absorption with heterologous fungal extracts sharing common antigens with *A. fumigatus* and/or *P. brasiliensis*. The antigen-antibody containing gels were then injected into adult rabbits by the method of Vaitukaitis, Robbins, Nieschlag & Ross (17). The animals were bled weekly and the sera containing antibodies against the specific antigens were stored at −20°C.

Serological Procedures

All the sera were simultaneously tested by the specific DDG technique (SDDT), by the classical DDG test (CDDT), and by the immunoelectrophoresis test (IEPT). For the SDDT one per cent agarose (Pastagarose, Institut Pasteur, Paris), in veronal buffer, pH:8·2, was employed. Samples of 4 ml of melted agarose were poured over microscopic slides and allowed to harden.

Three series of 3 mm. ∅ wells were cut with cork borers, the agarose being removed with a needle. Wells of the upper and lower series were filled with 15 microliter of undiluted human sera. Equal amounts of experimental monospecific antisera were employed to fill the wells of the middle series. The antigens were placed in 1 mm ∅ wells cut in the center of the triangular areas limited by the wells receiving the sera.

Approximately 2·5 microliter of a 50 mg/ml solution of the corresponding antigen was required to fill these wells. The diagram is schematized in Fig. 1A. Slides were utilized 30 minutes after preparation, and once used they were placed in a moist chamber and incubated at 25°C for 24 h and then, at 4°C for 48 h. After this incubation period, the slides were immersed in 5% sodium citrate solution for 1 h, rinsed in phosphate buffered saline pH:7·4 for 76 h, desmineralized, dried, and stained with amidoschwarz.

Two agarose-gel patterns were done (Fig. 1B). In pattern number one the well corresponding to the reference werum was filled with the rabbit antiserum anti-C_2 antigen of *A. fumigatus*, extract. In pattern number two, the wells of the middle line were filled with the antiserum anti-E_2 substance of *P. brasiliensis*, and the 1 mm

∅ wells, with crude extract of *P. brasiliensis*. In both patterns, the wells corresponding to the upper and lower series were filled with human sera. The classical DDT utilized was the one employed by Yarzábal *et al.* (21), in the case of aspergillosis, and that described by Restrepo (11) in paracoccidioidomycosis.

The immunoelectrophoresis test was carried out by the method already described (1).

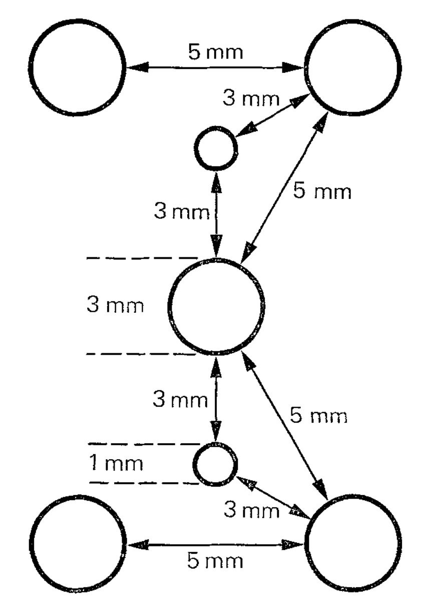


Figure 1.—A. Basic diagram of the SDDT

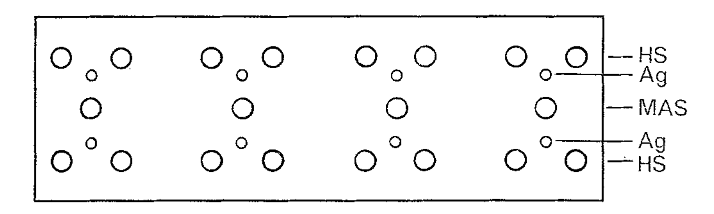


Figure 1.—B. Patterns for the SDDT. HS = human serum
Ag. = Crude antigen from either *A. fumigatus* or *P. brasiliensis*
MAS = Anti-C_2 or anti-E_2 monospecific antiserum

Results

All slides were read primarily after 24 h but a final reading was made on the sixth day after staining. No significant differences were detected between both readings. The results included here are those corresponding to the final reading.

Slides were immersed in sodium citrate with the aim to eliminate precipitating systems formed by C-reactive protein. Thus we may determine the real number

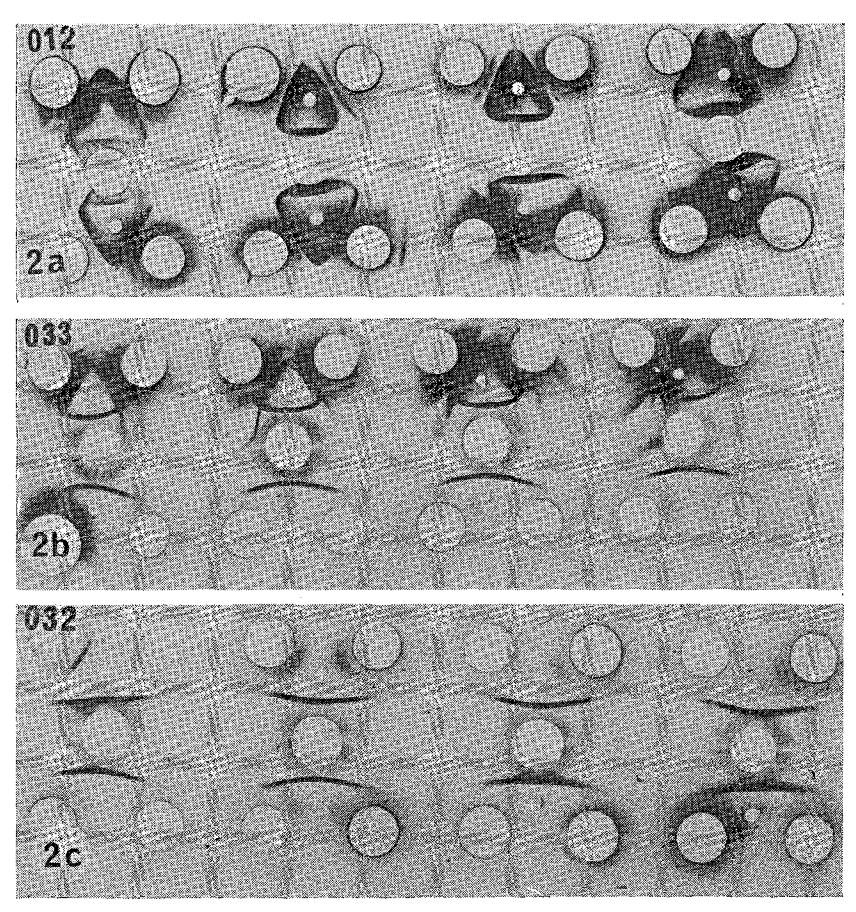


Figure 2.—A. Reaction of identity between human sera from paracoccidioidomycosis patients (wells of upper and lower series) and rabbit anti-E_2 antiserum (middle series), in the presence of crude extract of *P. brasiliensis* (small wells).
B. Reactions of identity between human sera from aspergillosis patients (all wells of upper and first of lower series) and rabbit anti-C_2 antiserum (middle series), in the presence of crude extract of *A. fumigatus* (small wells).
C. Non-identity reaction between human sera from paracoccidioidomycosis patients (wells 4 and 7 of upper series, and 7 of lower series), and rabbit anti-C_2 antiserum, in the presence of crude extract of *A. fumigatus* (small wells).

of antigen-antibody precipitating systems, an important data for the interpretation of the test.

(A) *Immunodiagnosis of aspergillosis*

As can be seen in Table 1, the homologous sera from the 9 cases of aspergilloma reacted positively in all the immunoprecipitation techniques employed in this study. The specific band C_2 revealed by the IEPT in all positive sera, was simultaneously identified by the SDDT. In 24 of the 121 heterologous sera, the CDDT exhibited non-specific reactivity; the SDDT and the IEPT remained negative. The sera showing antibodies against *Aspergillus* antigens other than C_2, belonged to the groups

of individuals affected by paracoccidioidomycosis (16/29), sporotrichosis (4/11), histoplasmosis (3/15) and candidiasis (1/5). Sera from healthy donors proved non-reactive in all three techniques.

(B) *Immunodiagnosis of Paracoccicioidomycosis*

As shown in Table 2, the SDDT and the IEPT gave specific precipiting-bands with 43 of the sera from paracoccidioidomycosis patients, while CDDT was reactive with 41. Statistical analysis showed that this difference was not significant.

TABLE 1.—COMPARATIVE SENSITIVITY AND SPECIFICITY OF THE SPECIFIC DOUBLE DIFFUSION TEST (SDDT), THE CLASSICAL DOUBLE DIFFUSION TEST (CDDT), AND THE IMMUNOELECTROPHORESIS TEST (IEPT), IN THE DIAGNOSIS OF ASPERGILLOSIS

Sera from patients with	*No. of subjects tested*	*Immunoprecipitation Techniques* *SDDT* +	*SDDT* −	*CDDT* +	*CDDT* −	*IEPT* +	*IEPT* −
Aspergilloma	9	9	0	9	0	9	0
Healthy donors	25	0	25	0	25	0	25
Heterologous controls							
Systemic Candidiasis	6	0	6	1	5	0	6
Chromomycosis	3	0	3	0	3	0	3
Coccidioidomycosis	4	0	4	0	4	0	4
Histoplasmosis	18	0	18	3	15	0	18
Lobomycosis	1	0	1	0	1	0	1
Mycetomas	4	0	4	0	4	0	4
Paracoccidioidomycosis	45	0	45	16	29	0	45
Sporotrichosis	15	0	15	4	11	0	15
Heterologous Controls Totals:	96	0	96	24	72	0	96

All the paracoccidiodiomydosis sera reactive in CDDT were detected by the SDDT and by the IEPT, but Restrepo's procedure showed 3 false positive results, 2 in the group with histoplasmosis and 1 in the group with aspergillosis. The serum of lobomycosis patient formed 2 precipitation bands in the SDDT, but neither one showed an identity reaction with that formed by the monospecific antiserum. The corresponding IEPT proved that these bands were different from band E and were distributed in the anodic region of the slide.

DISCUSSION

The results presented here show that the micro-double diffusion technique using experimental monospecific antisera may be a valuable tool for the diagnosis of both aspergillosis and paracoccidioidomycosis. The procedure requires only 15 microliter of the suspected serum and 2·5 microliter of a 50 mg/ml solution of the corresponding antigen. This implies a ten fold reduction in the volume of reactants consumed by the "classical" double diffusion test employed presently in the immunodiagnosis of both mycotic diseases. The sensitivity and the specificity of the

proposed technique are similar to those of the highly specific immunoelectrophoresis test. In the case of aspergillosis, the micro double diffusion test revealed antibodies against the specific C_2 antigen of *A. fumigatus* with the same frequency than the one observed with immunoelectrophoresis. Obviously, the test must be negative in: (i) *A. fumigatus* infections where anti-C_2 antibodies are not produced by the host and (ii) aspergillosis cases due to other species of the genus. In the first situation the immunoelectrophoresis test must be considered as the most efficient test since it is able to reveal the presence and permits the identification of antibodies formed against other specific antigens of *A. fumigatus* i.e. bands J, M and F(16). But these bands may be used in the future for the experimental production of monospecific antisera, thus being useful for the specific immunodiffusion test.

TABLE 2.—COMPARATIVE SENSITIVITY AND SPECIFICITY OF THE SPECIFIC DOUBLE DIFFUSION TEST (SDDT), THE CLASSICAL DOUBLE DIFFUSION TEST (CDDT), AND THE IMMUNOELECTROPHORESIS TEST (IEPT), IN THE DIAGNOSIS OF PARACOCCIDIOIDOMYCOSIS.

Sera from patients with	*No. of subjects tested*	*Immunoprecipitation Techniques*					
		SDDT		*CDDT*		*IEPT*	
		+	−	+	−	+	−
Paracoccidioidomycosis	45	43	2	41	4	43	2
Healthy donors	25	0	25	0	25	0	25
Heterologous controls							
Aspergilloma	9	0	9	1	8	0	9
Systemic Candidiasis	6	0	6	0	6	0	6
Chromomycosis	3	0	3	0	3	0	3
Coccidioidomycosis	4	0	4	0	4	0	4
Histoplasmosis	18	0	18	2	16	0	18
Lobomycosis	1	0	1	1	0	0	1
Mycetomas	4	0	4	0	4	0	4
Sporotrichosis	15	0	15	0	15	0	15
Heterologous Controls Totals:	60	0	60	4	56	0	60

In paracoccidioidomycosis the identification of a specific antigen-antibody system was possible for the first time in a double diffusion test. Characterization of band E confers to the double diffusion the specificity recognised for immunoelectrophoresis, without affecting the sensitivity of the double diffusion test. This is an important achievement because with a very simple method, a high level of specificity is attained, avoiding the inconveniences of immunoelectrophoresis. The close agreement between results obtained with the modified double diffusion technique and the immunoelectrophoresis test in this selected population suggests that the SDDT merits further application and evaluation as a diagnostic tool in the mycoses as well as in other infectious diseases.

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Resumen

Recurriendo al empleo de inmunosueros experimentales preparados en conejos contra antígenos especie-específicos de *Aspergillus fumigatus* (C_2) y de *Paracoccidioides brasiliensis* (E_2), se ha desarrollado una microtécnica simple y específica de doble difusión. El procedimiento fue evaluado en el inmunodiagnóstico de la aspergilosis y de la paracoccidioidomicosis. Sólo se consideraron positivos para aspergilosis o paracoccidioidomicosis, los sueros que produjeron líneas de identidad con las bandas formadas por los inmunosueros anti C_2, o anti E_2, respectivamente.

La sensibilidad diagnóstica de la prueba resultó similar a la de la doble difusión clásica e igual a la de la inmunoelectroforesis. La técnica no dio resultados falsos positivos con los sueros de pacientes afectados por otras micosis, ni con los de controles sanos. Las cantidades de reactivos requeridas para la microtecnica específica resultaron diez veces inferiores a las exigidas por la doble difusión clásica.

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